Effect of Mutations Downstream of the Internal Ribosome Entry Site on Initiation of Poliovirus Protein Synthesis

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Initiation of poliovirus translation is mediated by a large, structured segment of the 5' nontranslated region known as the internal ribosome entry site (IRES) and normally occurs 155 nucleotides (nt) downstream of the IRES at AUG₇₄₃ (the AUG at nucleotide 743). Functional AUG codons introduced at nt 611 or 614 reduced initiation at AUG₇₄₃ by 10 to 40% *in vitro* but had no effect on virus phenotype. To investigate the role of the nt 586-743 spacer in greater detail, four intervening termination codons were removed, and an additional AUG triplet at nt 683 was introduced by nucleotide substitution. Initiation at AUG₇₄₃ was reduced by only 50 to 80%, depending on the number of upstream initiation codons. Initiation at AUG₇₄₃ was also reduced following insertion of a stable hairpin at nt 630, but the reduction was modest in an ascites carcinoma cell extract. Initiation was more frequent at AUG₇₄₃ than at AUG₆₈₃ if mRNAs contained either an upstream initiation codon or the stable hairpin. These results suggested that not all initiation events at AUG₇₄₃ can be accounted for by a scanning-dependent mechanism. Translation of bicistronic mRNAs in which the intercistronic spacer contained nt 630 to 742 of the poliovirus 5' nontranslated region indicated that these residues are not able to act as an entry point for ribosomes independently of the IRES. Insertion of increasingly longer sequences immediately downstream of the stable hairpin progressively reduced initiation at AUG₇₄₃ without affecting initiation at AUG₆₈₃. These results are discussed in terms of a model for initiation of poliovirus translation in which a complex RNA superstructure upstream of nt 586 promotes ribosome binding at an entry point determined by specific downstream *cis*-acting elements.

The single-stranded RNA genomes of picornaviruses are functional mRNAs that are uncapped and have long (600 to 1,300-nucleotide [nt]) highly structured 5' nontranslated regions (NTRs) containing multiple AUG triplets (48). Initiation of translation of these mRNAs is cap independent and results from ribosomal entry into an internal segment of their 5' NTRs (2, 16, 31, 37). These properties distinguish picornavirus mRNAs from the majority of eukaryotic mRNAs, whose translation is dependent on the 5'-terminal cap and starts at the first AUG triplet of the RNA. Large segments of the 5' NTRs are required to mediate internal initiation: the internal ribosomal entry site (IRES) of encephalomyocarditis virus (EMCV) extends from nt 392 to 834 (18), while that of poliovirus extends from nt 134 to 585 (33). The sequences and secondary structures of the poliovirus IRES and the related human rhinovirus (HRV) elements differ from that of the EMCV IRES (32, 40, 41). A further notable difference is that whereas the 3' boundary of the EMCV IRES is at or close to the initiation codon (18), the 3' borders of the HRV and poliovirus IRES elements are separated from the upstream initiation codon by about 50 and 154 nt, respectively. However, a strikingly conserved motif is present in these and all other picornavirus IRES elements (17); a pyrimidine-rich (Y_n) tract ($n = 6$ to 9 nt) close to the IRES 3' boundary is separated by a nonconserved sequence (X_m, where $m = 15$ to 20 nt) from an AUG triplet. This AUG triplet is the 11th AUG in the EMCV 5' NTR and serves as the initiation codon for translation of the

EMCV polyprotein (47, 52). The corresponding AUG triplet at nt 586 (AUG₅₈₆) of the Y_n-X_m-AUG motif in poliovirus type 1 (Mahoney) [PV1(M)] RNA is the ninth AUG in the 5' NTR (21); it has a poor nucleotide context and does not serve as an initiation codon in translation (6). However, it does play a *cis*-acting role in IRES function and consequently in viral replication in cell culture (35, 39, 42). Moreover, alteration of the context of this triplet from CUUAUGG to ACCAUGG enables it to function as an initiation codon, an observation suggesting that translation of poliovirus RNA can commence at or immediately downstream of the 3' border of the IRES (39).

The sequence (or spacer) between the Y_n-X_m-AUG₅₈₆ motif at the 3' border of the IRES and the initiating AUG₇₄₃ codon has interesting properties. First, it harbors a small open reading frame (ORF) that varies in length, depending on the virus strain, but in which AUG₅₈₆ is always out of frame with AUG₇₄₃. In PV1(M), this reading frame overlaps the ORF of the polyprotein by 38 nt and has the potential to encode a polypeptide of 7.2 kDa (6). Second, the spacer contains a segment of some 100 nt whose sequence is the least conserved between the genomes of different poliovirus types and strains (43, 51). Interestingly, this spacer does not contain any AUG triplets, and insertion at nt 702 of a DNA segment containing an AUG triplet yielded a mutant virus exhibiting a small-plaque phenotype. This AUG triplet had undergone substitution or had been deleted entirely in pseudorevertant viruses that had large-plaque phenotypes (28, 29). These observations, together with the inhibition of translation that resulted from insertion of a stable hairpin at nt 631 (37), have led to the suggestion that the ribosome reaches the initiation codon at nt 743 [the ninth AUG of (PV1(M))] by binding to the IRES and then scanning the intervening spacer sequence (15). Several

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observations suggest that this genetic element has an important function(s). Most strikingly, the length of the spacer of 154 nt has been conserved among all poliovirus types and strains despite its sequence heterogeneity and even though poliovirus operates under conditions of genetic austerity (54). Moreover, replication of type 1 Sabin and Mahoney polioviruses lacking nt 600 to 727 in tissue culture is slightly impaired, and PV1(M) containing deletions within the spacer was significantly less neurovirulent than wild-type (*wt*) virus (13). Similar results have been reported concerning coxsackievirus type B1 (14). These observations suggest that the spacer contains *cis*-acting elements that could influence initiation of polyprotein translation at AUG₇₄₃.

We have investigated this possibility by placing AUG triplets at different positions within the intact spacer of PV1(M) and then determining the effects of these changes on translation from the authentic initiation codon at nt 743 and on poliovirus phenotype and genome structure during replication in tissue culture. We then assessed the ability of these triplets to serve as initiation codons in monocistronic and dicistronic mRNAs. Results obtained by using this approach indicate that initiation of poliovirus translation is more complex than suggested by existing models.

MATERIALS AND METHODS

Plasmid construction and site-directed mutagenesis. Site-directed substitutions were introduced into the 5' NTR of PV1(M) as previously described (38). Mutations were identified by sequence analysis using Sequenase as directed by the manufacturer (U.S. Biochemical). Mutagenic oligonucleotides are described according to the complementary sequence in PV1(M) and numbered according to its revised sequence (21). Those nucleotides that are not complementary to the *wt* sequence are underlined. The oligonucleotides were as follows:

- 1 (nt 605–624), 3' CAATAGTACCTCGCTTAACC 5'
- 2 (nt 601–624), 3' CTAACAATAGTATTACCTTAACC 5'
- 3 (nt 599–629), 3' GTCTACAATAGTACCTTAACCTAACC 5'
- 4 (nt 629–649), 3' CGGTAGGGCCTTTTCGCTCTG 5'
- 5 (nt 674–704), 3' GCGAGTTATACACACAAATTTGGTTCATG 5'

PV1(M) cDNA fragments containing nucleotide substitutions were cloned into plasmid pIRESP1 [renamed from pPV1(M) 5'-P1 (38)] by using standard procedures (44). pIRESP1 comprises nt 70 to 742 of the PV1(M) 5' NTR and the entire P1 capsid protein precursor coding region (nt 743 to 3380) followed by an opal (UGA) termination codon, under the transcriptional control of a T3 polymerase promoter.

New potential initiation codons were introduced into the 5' NTR with oligonucleotides 1, 2, 3, and 5. The nucleotides in the vicinity of the new triplet AUG₆₁₁ were changed from AUCAUAAAG (*wt*) to AUCAUGGAG by using oligonucleotide 1. The corresponding changes made with oligonucleotide 2 were from AUAAGCGA (*wt*) to AUAUGGGA and generated AUG₆₁₄. The new triplet at nt 614 generated with oligonucleotide 3 (AUCAUGGGA) was termed AUG_{614c} and has an upstream context that is similar to that of AUG₆₁₁. Mutations made by using oligonucleotide 5 simultaneously generated AUG₆₈₃ and replaced two triplets at nt 683 and 698 that would act as termination codons if translation were initiated at AUG₆₁₁ or AUG₆₁₄. This was done by changing the nucleotides CUCAUUGAGUGUGUUUACUCUAAGUA (*wt*) to CUCAUUGGUGUGUUUACUCCAAGUA. Similar potential termination codons at nt 638 and 644 were removed by using oligonucleotide 4, by changing the nucleotides CGGUGAAAGUGAGAC (*wt*) to CCGGGAAAGCGAGAC.

FIG. 1. Effects of upstream AUG triplets at either nt 611 or nt 614 on translation of a partial poliovirus polyprotein. (A) Schematic representation of a segment of the poliovirus 5' NTR, showing AUG triplets at nt 611 and 614 as open rectangles and showing AUG₅₈₆ (of the Y_n-X_m-AUG motif) as a smaller rectangle within the phylogenetically conserved hairpin (domain VI). Most of the IRES upstream of domain VI has been omitted. Downstream termination codons in frame with AUG₆₁₁ and AUG₆₁₄ are indicated by asterisks. ORFs and sizes of translation products initiated at AUG₆₁₁, AUG₆₁₄, and AUG₇₄₃ are shown below the schematic representation of the 5' NTR. (B) Translation of mRNA transcripts of pIRESP1 (*wt*) (lanes 1 to 5), pIRESP1(1) (which includes AUG₆₁₁) (lanes 6 to 10), and pIRESP1(2) (which includes AUG₆₁₄) (lanes 11 to 15) in a HeLa cell-free lysate. mRNAs were translated for 60 min 30°C over a range of RNA concentrations. Translation products were resolved by SDS-PAGE on a 10 to 20% gradient gel. The 97-kDa P1 translation product is indicated on the right. (C) Graphical representation of the efficiency of translation of the P1 partial polyprotein from pIRESP1 (*wt*) mRNA (□), pIRESP1(1) mRNA (●), and pIRESP1(2) mRNA (○) over a range of mRNA concentrations. Incorporation was determined by scanning laser densitometry of the gel in panel B and is in arbitrary units.

FIG. 2. One-step growth curves of wt PV1(M) (●) and mutant derivatives W1-5NC-4 (□) and W1-5NC-5 (◇) in HeLa S3 cells.

Substitutions made by using oligonucleotides 1 and 2 were introduced into pIRES1(1) and pIRES1(2), respectively, those made by using oligonucleotides 1, 4, and 5 were introduced into pIRES1(611), those made by using oligonucleotides 2, 4, and 5 were introduced into pIRES1(614), those made by using oligonucleotides 3, 4, and 5 were introduced into pIRES1(614c), and those made by using oligonucleotides 4 and 5 were introduced into pIRES1(683). Fragments of 864 nt were excised from between the *Avr*II sites in pIRES1, pIRES1(611), pIRES1(614), pIRES1(614c), and pIRES1(683) to generate plasmids pIRESΔP1, pIRESΔP1(611), pIRESΔP1(614), pIRESΔP1(614c), and pIRESΔP1(683), in which the largest ORF had been truncated so that it could, depending on initiation site, encode polypeptides of 20 to 25 kDa.

To insert a stable hairpin ($\Delta G_{37^\circ\text{C}} = -50$ kcal [ca. -209.2 kJ] mol⁻¹ [8]) between the IRES and the initiation codons at nt 683 and 743, a 39-nt fragment (5' GACGGTATCGATAAG CTTGATATCGAATTCCTGCAGCCC 3') was excised from the polylinker of pBS(SK⁺) (Stratagene, La Jolla, Calif.) by digestion with *Sma*I and *Hinc*II and inserted into the *Msc*I site (nt 630 of the 5' NTR) of plasmids pIRESΔP1, pIRESΔP1(611), and pIRESΔP1(683), generating plasmids pIRES(39⁺)ΔP1, pIRES(39⁻)ΔP1, pIRES(39⁺)ΔP1(611), pIRES(39⁻)ΔP1(611), pIRES(39⁺)ΔP1(683), and pIRES(39⁻)ΔP1(683). The notation (39⁺) indicates that the orientation of the insert is as described above; (39⁻) indicates the opposite orientation. Pairs of plasmids containing this insert in opposite orientations were digested with *Eco*RI, and fragments were exchanged to generate plasmids pIRES(stem)ΔP1 and pIRES(stem)ΔP1(683), which both contained the same ($\Delta G_{37^\circ\text{C}} = -209.2$ kJ mol⁻¹) 34 bp hairpin. The oligonucleotides 5' CCAATCTTGTCTGTTG GTGCTGGTGTTCAGGTC 3' and 5' GCACCTTGAAC ACCAGCACCAACAGAACAGATTGG 3' were annealed and inserted into the *Sma*I site of pIRES(stem)ΔP1(683), which corresponds to a position at the base of this hairpin. An insertion of 72 nt was present in plasmid pIRES(stem+72)ΔP1 (683).

Plasmids pΔP1 and pΔP1(683) were constructed by ligating the *Msc*I-*Eco*RI fragments derived from pIRESΔP1 and pIRESΔP1(683), respectively, between the *Hinc*II and *Eco*RI sites of pBluescript II (KS⁻) (Stratagene). mRNAs transcribed from these plasmids with T3 polymerase therefore contain only the last 113 nt of the PV1(M) 5' NTR (nt 630 to 742), which are not required for IRES function (33).

The triplets AUG₆₁₁ and AUG₆₁₄ were introduced into an infectious cDNA clone of poliovirus by exchanging the *Esp*I-*Sna*BI (nt 285 to 2953) fragment of plasmid T7-polio (46) for corresponding fragments from pIRES1(611) or pIRES1(614), yielding pT7[AUG₆₁₁] and pT7[AUG₆₁₄]. As a control, the chimeric wt plasmid pT7[XLG] was derived from pIRES1 and T7-polio in the same way.

In vitro transcription and translation. Plasmids were linearized by digestion with *Spe*I, *Bst*EII, or *Eco*RI (nt 2640, 3234, and 3386) as appropriate, transcribed with T3 or T7 RNA polymerase, and purified as described previously (4, 38). Synthetic mRNAs were translated in rabbit reticulocyte lysate (RRL; Promega Biotec), HeLa cell extract, or Krebs 2 cell extract in the presence of [³⁵S]methionine for 60 min at 30°C. Cell extracts from HeLa S3 and Krebs 2 cells were prepared and used as described previously (37, 49). Translation was done in 25 μl aliquots of cell-free lysate, using mRNA over a range of different concentrations. The resulting products were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using buffer conditions as described elsewhere (34). Translation efficiencies were quantitated by scanning X-ray films with an LKB laser densitometer and were normalized by assuming total removal of the initiating formyl-methionyl moiety; translation products resulting from initiation at AUG₆₁₁, AUG₆₁₄, AUG₆₈₃, and AUG₇₄₃ therefore contain five, five, four, and three methionine residues, respectively. β-Globin contains one methionine residue.

Characterization of poliovirus 5' NTR mutants. Full-length RNAs transcribed from pT7[XLG], pT7[AUG₆₁₁], and pT7[AUG₆₁₄] were transfected into HeLa R19 cells (53). Cells were harvested with the culture medium as soon as cytopathic effects became apparent, and viruses were plaque purified three times. High-titer stocks of a representative of each mutant virus and of wt viruses were purified after amplification in 1 liter of HeLa S3 suspension cells and used for all further characterization. Viral RNA was prepared, and its nucleotide sequence in the vicinity of the AUG codons was determined by the dideoxynucleotide method (7). Viral protein synthesis and virus production were examined at different times postinfection in duplicate monolayers of HeLa R19 cells, which were infected at a multiplicity of infection of 25 PFU per cell. Virus yields were determined by plaque assay, and protein synthesis was examined by pulse-labelling with a mixture of [³⁵S]methionine and [³⁵S]cysteine for 15 min at appropriate time intervals postinfection, after which aliquots of cell lysates were prepared for SDS-PAGE.

RESULTS

Influence of AUG triplets downstream of the IRES on initiation at AUG₇₄₃ in vitro and poliovirus replication in tissue culture. AUG triplets were introduced at two positions within domain VI of the PV1(M) 5' NTR to investigate whether initiation could occur between the IRES and AUG₇₄₃. The triplet AUG₆₁₁ was chosen to mimic the position of the HRV-2 initiation codon within domain VI and relative to the Y_n-X_m-AUG motif. AUG₆₁₄ is located 3 nt downstream of this position, and both triplets have a favorable context for initiation (AUCAUGG and AUAAUGG, respectively). These sub-

FIG. 3. Translation of poliovirus mRNA containing multiple upstream AUG triplets in the same ORF as the authentic initiation codon AUG₇₄₃. (A) Schematic representation of a segment of the poliovirus 5' NTR showing AUG triplets at nt 611, 614, 683, and 743 and corresponding methionine residues as black rectangles and showing AUG₅₈₆ as a smaller unfilled rectangle within a phylogenetically conserved hairpin (domain VI) at the 3' border of the IRES. Most of the IRES upstream of domain VI has been omitted. The context of potential initiation codons, corresponding ORFs, and sizes of potential translation products are shown below the schematic representation of the 5' NTR. (B and C) Translation of mRNA transcripts of pIRESΔP1 (lanes 1 to 5), pIRESΔP1(683) (lanes 6 to 10), pIRESΔP1(614) (lanes 11 to 15), and pIRESΔP1(611) (lanes 16 to 20) in HeLa (B) and Krebs 2 ascites carcinoma (C) cell extracts. mRNAs were translated for 60 min at 30°C at RNA concentrations that increased from 10 μg/ml (lanes 1, 6, 11, and 16) to 160 μg/ml (lanes 5, 10, 15, and 20) in twofold increments. Translation products were resolved by SDS-PAGE on a 10 to 20% gradient gel. The 25.1-, 22.5-, and 20.2-kDa translation products are indicated on the right.

stitutions do not significantly disrupt the base pairing within the conserved hairpin of domain VI (32, 40). Transcripts derived from pIRES_{P1}(1), pIRES_{P1}(1), and pIRES_{P1}(2) were translated over a range of concentrations in RRL and in HeLa and Krebs 2 cell extracts. The presence of the potential initiation codons AUG₆₁₁ and AUG₆₁₄ reduced the efficiency of translation in a HeLa cell lysate by about 40 and 10%, respectively (Fig. 1). Similar results were obtained with RRL and a Krebs 2 cell extract (data not shown).

Initiation of translation of the P1 polypeptide at nt 743 was impaired but not abolished by the introduction of functional AUG codons at nt 611 and 614. Two mutant viruses (W1-

5NC-4 and W1-5NC-5) were generated by transfection of mutated full-length RNAs into HeLa cells; they contained the sequences AUCAUGG(AUG₆₁₁) and AUA AUGG(AUG₆₁₄), respectively (data not shown), and one-step growth curves showed that their growth rates did not differ significantly from those of wt virus (Fig. 2). Plaques formed by these mutant viruses in HeLa R19, RD, CV-1, and Hep-2 cells were similar to those formed by wt virus (data not shown).

Introduction of AUG codons upstream of AUG₇₄₃ and in the same ORF. Translation of the P1 ORF was impaired but not abolished by the AUG triplets at nt 611 or 614 that could potentially initiate translation of short (eight- or nine-codon)

TABLE 1. Efficiency of initiation at AUG codons downstream of the poliovirus IRES

Cell-free lysate	mRNA	Initiation codon utilization ^a (%)			
		AUG ₆₁₁	AUG ₆₁₄	AUG ₆₈₃	AUG ₇₄₃
Krebs	pIRESΔP1(683)			58	47
	pIRESΔP1(614)		48	28	36
	pIRESΔP1(614c)		47	27	35
	pIRESΔP1(611)	77		12	19
HeLa	pIRESΔP1(683)			40	53
	pIRESΔP1(614)		31	25	39
	pIRESΔP1(614c)		33	22	41
	pIRESΔP1(611)	54		6	18

^a Translation products were quantitated by scanning X-ray films with an LKB laser densitometer. Efficiency of initiation is described relative to initiation at AUG₇₄₃ on translation of pIRESΔP1 mRNA, which was defined as 100%.

ORFS in the intact 5' NTR. Initiation of P1 translation at the downstream codon AUG₇₄₃ could have resulted from termination-reinitiation, leaky scanning, or bypassing of the triplets AUG₆₁₁ and AUG₆₁₄. To distinguish between these possibilities, the four in-frame termination codons downstream of nt 611 were altered by nucleotide substitution, which simultaneously generated AUG₆₈₃ (Fig. 3A). RNAs were transcribed from these plasmids that contained AUG₇₄₃ and either one or two upstream in-frame AUG triplets (AUG₆₈₃ alone or in combination with AUG₆₁₁, AUG₆₁₄, or AUG_{614c}). The P1 ORF was truncated to differentiate products initiated at these codons and to facilitate quantitation.

Initiation of translation at AUG₇₄₃ should yield a truncated 20.2-kDa form of P1, and this polypeptide was translated from *wt* RNA in HeLa and Krebs 2 cell extracts (Fig. 3B and C, lanes 1 to 5). Translation of mRNA containing AUG₆₈₃ and AUG₇₄₃ yielded this 20.2-kDa polypeptide and a 22.5-kDa polypeptide that presumably resulted from initiation at AUG₆₈₃ (Fig. 3B and C, lanes 6 to 10). Initiation occurred a little more frequently (ca. 35%) at AUG₇₄₃ than at AUG₆₈₃ in the HeLa cell lysate; this ratio was reversed on translation in a Krebs 2 cell extract (Table 1). The combined frequency of initiation at AUG₆₈₃ and AUG₇₄₃ was similar in both cell-free lysates to that of initiation at AUG₇₄₃ alone upon translation of *wt* mRNA.

The predominant product translated from pIRESΔP1(611) mRNA in Krebs 2 and HeLa cell extracts was a 25.1-kDa polypeptide (presumably resulting from initiation at AUG₆₁₁) (Fig. 3B and C, lanes 16 to 20). The 22.5- and 20.2-kDa polypeptides were also apparent, and significantly, translation of the latter was three times more frequent than of the former (Table 1). Striking changes in the frequency of initiation codon selection within the ΔP1 ORF resulted from the introduction of AUG₆₁₄ in place of AUG₆₁₁. AUG₆₁₄ is immediately downstream and adjacent to AUG₆₁₁ and has a context (AUAAUGG) that is not obviously worse than that of AUG₆₁₁ (AUACAUGG). However, initiation from AUG₆₁₄ was much less frequent than from AUG₆₁₁ in both HeLa and Krebs 2 cell-free lysates (Fig. 3B and C, lanes 11 to 15; Table 1). Alteration of the context of AUG₆₁₄ (AUAAUGG) to AUG_{614c} (AUACAUGG) so that it resembled AUG₆₁₁ at all positions from -3 to +4 did not alter the frequency of utilization of this codon (Table 1). Initiation at AUG₆₁₁ was reduced upon insertion of 39 nt at nt 630 in pIRES(39⁺)ΔP1 mRNA, indicating that initiation at this codon can be influenced by downstream elements (data not shown). The combined frequency of initiation at the three AUG codons in pIRESΔP1(614) and pIRESΔP1(611) mRNAs was similar to

TABLE 2. Effects of nucleotide insertions at nt 630 on the efficiency of initiation at AUG codons downstream of the poliovirus IRES

Cell-free lysate	mRNA	Initiation codon utilization ^a (%)	
		AUG ₆₈₃	AUG ₇₄₃
Krebs	pIRES(stem)ΔP1		74
	pIRES(stem)ΔP1(683)	27	42
	pIRES(stem+13nt)ΔP1(683)	32	32
	pIRES(stem+19nt)ΔP1(683)	31	29
	pIRES(stem+72nt)ΔP1(683)	32	22
	pIRES(stem)ΔP1		16
HeLa	pIRES(stem)ΔP1(683)	4	12
	pIRES(stem+72nt)ΔP1(683)	5	4

^a Translation products were quantitated by scanning X-ray films with an LKB laser densitometer. Efficiency of initiation is described relative to initiation at AUG₇₄₃ on translation of pIRESΔP1 mRNA, which was defined as 100%.

that of initiation at AUG₇₄₃ alone on translation of *wt* mRNA (Table 1), and initiation at AUG₆₈₃ and AUG₇₄₃ was therefore inversely related to the efficiency of initiation at the upstream codon within domain VI in these mRNAs. None of the four upstream codons (AUG₆₁₁, AUG₆₁₄, AUG_{614c}, or AUG₆₈₃) prevented initiation at the *wt* codon AUG₇₄₃, and initiation at AUG₇₄₃ represented between 17.5 and 59% of total initiation events that occurred during translation of this group of mRNAs containing one or more functional upstream AUG codons.

Influence of a stable hairpin downstream of the IRES on AUG codon utilization. Results presented above indicate that initiation at AUG₇₄₃ on mRNAs containing upstream initiation codons is not solely due to termination-reinitiation. Initiation at AUG₇₄₃ could have resulted from leaky scanning, and in order to discriminate between this and alternative mechanisms, a stable hairpin ($\Delta G_{37^\circ\text{C}} = -209.2 \text{ kJ mol}^{-1}$) was inserted into the 5' NTR at nt 630, at the apex of the loop in domain VII (10). Insertion of this hairpin would extend the helical stem in domain VII from 7 nt to 34 nt. This modification was made because insertion of a similar hairpins into the 5' NTR of an mRNA has been shown to reduce initiation at a downstream AUG codon by 85 to 95% (25, 36). Hairpins may act as barriers to scanning ribosomes, and their inhibitory effect has been taken as evidence for the linearity of scanning. A stable hairpin inserted between the 3' border of the IRES and AUG₆₈₃ would therefore be expected to exert an inhibitory effect on initiation at AUG₆₈₃ and AUG₇₄₃ if initiation at the latter codon normally involved scanning. Appropriate derivatives of plasmids pIRESΔP1 and pIRESΔP1(683) were therefore constructed and termed pIRES(stem)ΔP1 and pIRES(stem)ΔP1(683), respectively (Fig. 4A).

The presence of this hairpin reduced initiation at AUG₇₄₃ on translation of pIRES(stem)ΔP1 mRNA in Krebs 2 and HeLa cell extracts to 74 and 16% of *wt* levels, respectively (Fig. 4B and C, lanes 1 to 10; Table 2). The reduction in the HeLa extract is consistent with a previous report (37), but the relatively small inhibition of translation in the Krebs 2 extract was unexpected. The presence of this hairpin also reduced the combined frequency of initiation at AUG₆₈₃ and AUG₇₄₃ on translation of mRNAs containing both codons by similar proportions (Table 2). This reduction was mostly due to reduced initiation at AUG₆₈₃, particularly in the Krebs 2 cell extract (Fig. 4B; compare lanes 11 to 15 with lanes 16 to 20), and as a result, the ratio of initiation events at these two codons was altered in favor of the downstream codon AUG₇₄₃

FIG. 4. Effect of inserting a stable hairpin at nt 630 of the poliovirus 5' NTR on initiation of translation at AUG₆₈₃ and AUG₇₄₃. (A) Schematic representation of pIRESΔP1(683) and pIRES(stem)ΔP1(683) mRNAs, showing IRES elements as zigzag lines, the hairpin at nt 630, AUG₆₈₃ as a black circle, and AUG₇₄₃ as a black rectangle. The context of potential initiation codons, corresponding ORFs, and sizes of translation products are shown below the schematic representation of the mRNAs. (B and C) Translation of mRNA transcripts of pIRESΔP1 (lanes 1 to 5), pIRES(stem)ΔP1 (lanes 6 to 10), pIRESΔP1(683) (lanes 11 to 15), and pIRES(stem)ΔP1(683) (lanes 16 to 20) in Krebs 2 ascites carcinoma (B) and HeLa (C) cell extracts. mRNAs were translated for 60 min at 30°C at RNA concentrations that increased from 10 μg/ml (lanes 1, 6, 11, and 16) to 160 μg/ml (lanes 5, 10, 15, and 20) in twofold increments. Translation products were resolved by SDS-PAGE on a 10 to 20% gradient gel. The 22.5- and 20.2-kDa translation products are indicated on the right.

by a greater than twofold factor. It is possible that the hairpin altered the secondary structure of the spacer so as to preclude initiation at AUG₆₈₃. However, introduction of the hairpin in conjunction with AUG₆₈₃ had an effect on initiation at AUG₇₄₃ very similar to that caused by the hairpin alone, and we consider it more likely that both changes affect the same stage in initiation, which is probably scanning (25, 36). A proportion of initiation events at AUG₇₄₃ appears to have resulted from an alternative mechanism, and additional

changes to the poliovirus 5' NTR were therefore made to test two potential mechanisms for initiation at this codon.

Effect of increasing the length of the spacer between the IRES and AUG₇₄₃. Translation of bicistronic mRNAs confirmed that the observations reported above concerning initiation codon selection were the consequence of internal ribosomal entry and indicated that residues downstream of nt 630 cannot act as an entry point for ribosomes independently of the IRES (data not shown). A second mechanism, involving ribo-

FIG. 5. Effect of inserting random nucleotides immediately downstream of the stable hairpin in pIRES(stem)ΔP1(683) mRNA on initiation at AUG₆₈₃ and AUG₇₄₃. (A) Schematic representation of pIRES(stem)ΔP1(683) mRNA and the mRNAs pIRES(stem+13nt)ΔP1(683), pIRES(stem+19nt)ΔP1(683) mRNA and pIRES(stem+72nt)ΔP1(683) derived from it, showing the IRES as a bold zigzag line, the hairpin at nt 630, AUG₆₈₃ as a black circle, AUG₇₄₃ as a black rectangle, the ΔP1 coding sequence, and the sequences of nucleotides inserted immediately

somal entry promoted by the IRES at positions downstream of nt 586, possibly followed by 5'-3' scanning, was therefore considered. We reasoned that if reduced initiation caused by the hairpin at nt 630 were due to reduced access of ribosomal subunits to the segment of the spacer between nt 630 and 743, then insertion of residues into this segment should restore the level of initiation at AUG₆₈₃. Derivatives of the plasmid pIRES(stem) Δ P1(683) that contained insertions of 13, 19, or 72 nt adjacent to and immediately downstream of the hairpin at nt 630 were therefore constructed (Fig. 5A). The longest insert places AUG₆₈₃ at a distance from the 3' border of the IRES that is similar to that of AUG₇₄₃ in mRNA transcribed from pIRES(stem) Δ P1(683).

These changes had no effect initiation at AUG₆₈₃ in either murine ascites carcinoma or HeLa cell extracts, but initiation at AUG₇₄₃ was progressively reduced as the length of the insert was increased (Fig. 5B and C; Table 2). Similarly, insertion of 72 nt downstream of the hairpin did not affect initiation at AUG₆₈₃ on translation in rabbit reticulocyte lysate but halved initiation at AUG₇₄₃ (data not shown). These results do not support a mechanism for initiation at AUG₇₄₃ that involves IRES-promoted entry of ribosomal subunits into the spacer at random positions downstream of the hairpin at nt 630. However, they do not rule out the possibility that the IRES could promote ribosomal entry at a specific downstream site (such as in the immediate vicinity of AUG₇₄₃) possibly by means of a looping-out mechanism.

Initiation codon selection in the absence of the poliovirus IRES. The efficiency of translation of truncated monocistronic poliovirus mRNAs containing nt 630 to 742 of the 5' NTR, AUG₆₈₃, and/or AUG₇₄₃ in Krebs 2 and HeLa cell lysates was lower than that of mRNAs containing the intact IRES but was restored by capping these truncated transcripts (Fig. 6). The ratio between initiation events occurring at nt 683 and at nt 743 was not dependent on the presence of the IRES or a 5'-terminal capping group in the Krebs 2 cell lysate; surprisingly, initiation at AUG₇₄₃ occurred more frequently than initiation at AUG₆₈₃ when mRNAs lacking the IRES were translated in HeLa cell-free lysate. This result indicates that preferential utilization of AUG₇₄₃ is independent of the mechanism of ribosomal attachment to mRNA.

DISCUSSION

The initiation codon of the poliovirus polyprotein at AUG₇₄₃ is separated from the 3' border of the IRES by a 154 nt spacer which may contain *cis*-acting elements that influence the efficiency of translation (13, 14, 33, 39). We have altered the sequence and structure of this spacer to investigate its influence on initiation and to assess the role of scanning in poliovirus translation. Current models for poliovirus translation suggest that initiation at AUG₇₄₃ involves ribosome scanning of the spacer after entry at the 3' border of the IRES (reviewed in references 12 and 15). Some of the observations reported here are compatible with this model: AUG triplets placed downstream of the ribosome entry point acted as initiation codons and reduced initiation at AUG₇₄₃, and inser-

tion of a stable hairpin at nt 630 reduced initiation at downstream AUG codons, including AUG₇₄₃. These results are characteristic of those used to develop the model of ribosome scanning (23-27). However, other results presented here are difficult to reconcile with a conventional scanning-dependent mechanism of initiation at AUG₇₄₃, for which reason we have considered alternative mechanisms for IRES-dependent initiation.

We first noted that the initiation codons AUG₆₁₁ and AUG₆₁₄ were retained in mutant polioviruses and had no apparent effect on virus phenotype. An equally unexpected observation was that the presence of upstream initiation codons reduced initiation at AUG₇₄₃ by only 50 to 80%, which is less than expected for a conventional scanning mechanism (24, 27). Moreover, initiation at AUG₇₄₃ was up to three times more frequent than initiation at AUG₆₈₃ when mRNAs that contained a third initiation codon at either AUG₆₁₁ or AUG₆₁₄ were translated. These upstream codons and AUG₇₄₃ all have similar favorable contexts for initiation, and it is therefore not easy to reconcile these results with leaky scanning because upstream initiation codons are normally bypassed only if they occur very close to the ribosome entry point or have a poor context. Insertion of a stable hairpin at nt 630 reduced initiation at AUG₇₄₃ sixfold in a HeLa cell-free lysate, which is consistent with an earlier report (37), but had a much smaller effect on translation in a Krebs 2 cell extract. Unexpectedly, insertion of the same hairpin into mRNA containing tandem initiation codons reduced initiation at AUG₇₄₃ two to three times less than at AUG₆₈₃, a result observed in both HeLa and ascites carcinoma cell extracts. Studies on eukaryotic cap-dependent translation have shown that the progress of scanning ribosomes is impeded by the presence of a stable hairpin within the 5' NTR, resulting in reduced initiation at a downstream codon (25, 36). Initiation at AUG₆₈₃ was more sensitive to inhibition by upstream AUG triplets and/or stable secondary structures than initiation at AUG₇₄₃, so the activity of this initiation codon may be more dependent on scanning than initiation at AUG₇₄₃. Consequently, the relative insensitivity of initiation at AUG₇₄₃ to inhibition by these elements must reflect the contribution of a second mechanism to initiation.

Cap-dependent scanning is conventionally thought of in terms of threading a needle (27), but the fact of ribosomal entry into an internal RNA segment indicates that the mRNA track on the surface of the 40S ribosomal subunit may not be constrained (9). A model for IRES-dependent initiation in which a complex RNA structure upstream of nt 586 directs ribosomal binding to a downstream entry point is therefore feasible and would enable ribosomes to bypass hairpins and initiation codons sequestered in looped-out sequences upstream of AUG₇₄₃. Ribosomes bypassed the stable hairpin at nt 630 with higher efficiency in Krebs 2 cell extracts than in HeLa cell extracts (Table 2). The proposed scanning-independent mechanism may therefore involve tissue-specific *trans*-acting factors; it clearly depends on the structural integrity of the region downstream of the hairpin. The presence of increasingly long inserts downstream of the hairpin had no effect on

downstream of the hairpin. The context of potential initiation codons, corresponding ORFs, and sizes of translation products are shown below the schematic representation of the mRNAs. (B) Translation of mRNA transcripts of pIRES(stem) Δ P1(683) (lanes 1 to 5, 16 to 20, and 26 to 30), pIRES(stem+13nt) Δ P1(683) (lanes 6 to 10), pIRES(stem+19nt) Δ P1(683) (lanes 11 to 15), and pIRES(stem+72nt) Δ P1(683) (lanes 21 to 25 and 31 to 35) in Krebs 2 ascites carcinoma (lanes 1 to 25) and HeLa (lanes 26 to 35) cell extracts. mRNAs were translated for 60 min at 30°C at RNA concentrations ranging from 5 μ g/ml (lanes 1, 6, 11, 16, 21, 26, and 31) to 80 μ g/ml (lanes 5, 10, 15, 20, 25, 30, and 35) in twofold increments. Translation products were resolved by SDS-PAGE on 10 to 20% gradient gels. The 22.5- and 20.2-kDa translation products are indicated on the right.

FIG. 6. Influence of the IRES on initiation of poliovirus translation at AUG₆₈₃ and AUG₇₄₃. (A) Schematic representation of pIRES-ΔP1(683) and pΔP1(683) mRNAs, showing the 5' terminus as a black triangle, the IRES as a bold zigzag line, the downstream spacer as a straight boldface line, AUG₆₈₃ as a black circle, and the ΔP1 cistron as a stippled rectangle. (B and C) Translation of mRNA transcripts of pIRESΔP1(683) (lanes 1 to 4), uncapped transcripts of pΔP1(683) (lanes 5 to 8), and capped transcripts of pΔP1(683) (lanes 9 to 12) in cell extracts of HeLa (B) and Krebs 2 ascites carcinoma (C) cells. mRNAs were translated for 60 min at 30°C over a range of RNA concentrations that increased in twofold increments. Translation products were resolved by SDS-PAGE on 10 to 20% gradient gels. The 22.5- and 20.2-kDa translation products are indicated on the right.

initiation at AUG₆₈₃ but progressively reduced initiation at AUG₇₄₃, even though the longest insert placed AUG₆₈₃ at a distance downstream of the hairpin similar to that between AUG₇₄₃ and the hairpin in the original construct pIRES (stem)ΔP1 (Fig. 5). These results are not consistent with scanning from the 3' border of the IRES but could result from disruption of a downstream *cis*-acting element. This model would account for the observation that insertion of a 72-nt sequence containing an AUG triplet at nt 702 impaired poliovirus replication (28), whereas the substitutions in the mutants W1-5NC-4 and W1-5NC-5 caused minimal structural perturbation and did not affect virus growth.

Two experimental observations support a model in which IRES-dependent initiation depends on a complex upstream RNA structure and on noncontiguous downstream *cis*-acting elements and indicate that these components can be separated without complete loss of function. First, insertion of the complete influenza virus NS coding region at nt 630 in the poliovirus 5' NTR did not abolish initiation at AUG₇₄₃ (12). Second, initiation of *wt* EMCV translation occurs specifically at AUG₈₃₄ and bypasses AUG₈₂₆ completely, although it has a favorable context (20, 47, 52). Insertion of 8 nt at nt 805 did not

result in significant initiation at AUG₈₂₆ at the expense of AUG₈₃₄, indicating that selection of the latter triplet as the preferred initiation codon depends on more than simply its distance downstream from the IRES, and may also be scanning independent (19). In this model, the activity of the entry point at which ribosomes bind would depend on the upstream RNA structure that promotes ribosome entry but not vice versa. A mutation in the upstream structure might therefore inhibit IRES function, whereas a mutation within a downstream *cis*-acting element might simply result in utilization of an alternative (lower-affinity) entry point. The observation that mutations that reduced initiation at EMCV AUG-11 resulted in initiation at three distinct downstream AUG codons (rather than at the single site that would have been predicted from a simple entry/scanning model) is consistent with this proposal (5). Moreover, the model provides a rationale for initiation at multiple AUG triplets, which is characteristic of many picornavirus IRES elements, and it may account for the observation that initiation occurs at the second, downstream codon in Theiler's murine encephalitis virus and in some foot-and-mouth disease virus strains despite the first upstream codon having a good context (3, 22, 45, 50). Initiation at the down-

stream codon of both hepatitis A virus and foot-and-mouth disease virus is enhanced by their respective IRES elements, varies between cell types, and depends on local nucleotide context and on the endogenous magnesium concentration (1, 3, 45, 50).

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